

## The Mouse Fas-ligand Gene is Mutated in *gld* Mice and Is Part of a TNF Family Gene Cluster

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### Summary

The gene for the mouse *Fas* ligand was cloned and its chromosomal position determined. *FasL* was tightly linked to *gld* (no crossovers in 567 meiotic events) on mouse chromosome 1 and closely linked with a novel member of the same TNF family of ligands, the Ox40 ligand (Ox40L, 1 crossover in 567 meiotic events). Southern blot analysis did not reveal any difference between the *FasL* gene from *gld* and wild-type mice and levels of *FasL* mRNA transcripts were similar in PMA and ionomycin induced wild-type and cologenic *gld* T cells. Sequence analysis of the *gld* gene indicated a single amino acid change (Phe → Leu) in the COOH terminal portion of this type II transmembrane protein, and COS cells transfected with *FasL* cDNA from *gld* mice failed to induce apoptosis of *Fas*-expressing target cells. Thus, the data demonstrate that the *gld* phenotype is the result of a point mutation in the *FasL* gene and that *FasL* is part of a complex of ligands structurally related to TNF mapping within a small region of mouse chromosome 1.

### Introduction

*Fas* is a member of a family of cysteine-rich transmembrane proteins that includes the type I and type II tumor necrosis factor (TNF) receptors, the low affinity nerve growth factor receptor, CD27, CD30, CD40, 4-1BB and Ox40 (Camerini et al., 1991; Dürkop et al., 1992; Itoh et al., 1991; Johnson et al., 1986; Kwon and Weissman, 1989;

Loetscher et al., 1990; Mallet et al., 1990; Schall et al., 1990; Smith et al., 1990; Stamenkovic et al., 1989). The *Fas* protein was originally identified by two MAbs (Fas[CH-11] and APO-1) that were found to induce apoptosis in certain transformed cell lines expressing *Fas* in vitro (Itoh et al., 1991; Trauth et al., 1989). It is also possible that the *Fas* protein may play an important role in the activation and proliferation of normal T cells, since treatment of fresh peripheral blood T cells with *Fas*-specific MAb provides a potent costimulatory signal (Alderson et al., 1993).

The fact that mice with defective *Fas* genes (*lpr* and *lpr<sup>g</sup>*) develop a progressive autoimmune disorder indicates that this cell-surface receptor plays an important biological role in vivo (Watanabe-Fukunaga et al., 1992). Interestingly, the autoimmune syndrome that occurs in mice homozygous for the *gld* gene is remarkably similar to that observed in mice homozygous for the *lpr* gene (Cohen and Eisenberg, 1991). However, the *gld* gene maps to the distal portion of mouse chromosome 1 between the *At-3* and *Elam* genes (Seldin et al., 1988; Watson et al., 1992a), whereas the *lpr* gene maps to mouse chromosome 19 (Watanabe et al., 1991; Watson et al., 1992b). These observations, together with the results of bone marrow transplantation studies, suggest that the *lpr* and *gld* genes encode an interacting receptor-ligand pair (Allen et al., 1990). This hypothesis is supported by recent studies that demonstrate that *gld/gld* mice do not express a functional ligand for *Fas* (*FasL*) upon stimulation with phorbol ester and calcium ionophore, whereas wild-type mice and *lpr/lpr* mice do (Ramsdell et al., 1994; and P. Golstein, personal communication).

### Results

#### Cloning of the Mouse *FasL* Gene

To determine whether the *gld* phenotype is due to a structural or functional mutation in the *FasL* gene, mouse *FasL* genes from both wild-type and *gld/gld* mice were cloned using a polymerase chain reaction (PCR)-based strategy with primers complementary to base pairs 683-705 and 839-862 of the rat *FasL* sequence (Suda et al., 1993). PCR amplification using a template of cDNA prepared from a CD6<sup>+</sup> mouse cytotoxic T lymphocyte (CTL) line of C57BL/10SnJ origin induced to express *FasL* yielded the predicted 180 bp product, which was then used to screen a cDNA library prepared from these CTL. Plaque hybridization of approximately 200,000 clones resulted in several positive clones, three of which were chosen for further analysis. Clones *mFasL-1*, *mFasL-2*, and *mFasL-3* contained inserts of 1.7 kb, 1.6 kb, and 1.0 kb, respectively. Sequence analysis of the three clones revealed that the 1.6 kb and 1.0 kb clones were completely contained within the 1.7 kb insert, and all three clones shared a common polyadenylation site. The nucleotide sequence and predicted amino acid sequence of the mouse *FasL* is shown in Figure 1. Analysis of the coding region of clone *mFasL-1* revealed a 91% identity with the rat homolog at the amino acid level.

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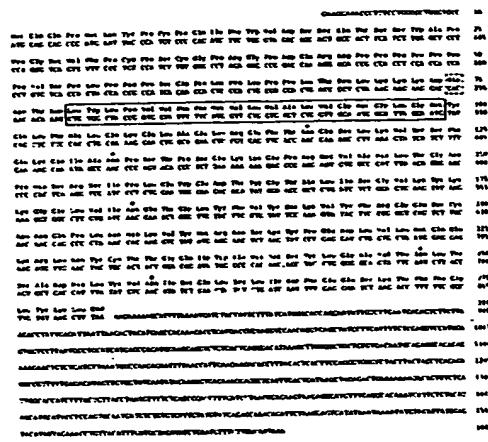


Figure 1. Nucleotide Sequence and Predicted Amino Acid Sequence of Mouse FasL.

The codon for the unique cytoplasmic histidine residue not detected in the sequence of rat FasL is indicated with a broken box. The putative transmembrane domain is boxed and five potential N-linked glycosylation sites (N-X-S/T) are indicated by asterisks.

Interestingly, one difference in the sequence of mouse FasL when compared with rat FasL was the insertion of a CAC codon at nucleotide 253, which results in an in-frame insertion of a histidine in the putative intracytoplasmic portion of the molecule. The nucleotide sequence of the coding region of FasL of C3H/HeJ origin was determined to be identical with that of B10 origin (data not shown).

#### Chromosomal Mapping of Mouse FasL

The chromosomal location of the FasL gene was determined using an interspecific cross [(C3H-gld × Mus spretus) F1 × C3H-gld] that had been characterized for markers that span each mouse autosome and the X chromosome. Using the BglII restriction endonuclease, an informative restriction fragment length polymorphism (RFLP) was identified that distinguished the two parents used in this cross (C3H/HeJ-gld, 7.5 kb; Mus spretus, 2.1 kb) (Figure 2A). Analysis of RFLPs in the backcross panel indicated that the FasL gene mapped to the region of mouse chromosome 1 that contained the gld mutation. In each of 567 backcross DNA samples from gld phenotype-positive mice, the homozygous C3H-gld pattern indicated that the FasL locus cosegregated with the gld phenotype on distal mouse chromosome 1 (Figure 2B). Interestingly, a novel member of the TNF family of ligands, the Ox40L, has recently been isolated by expression cloning using an Ox40Fc fusion protein (P. R. B., unpublished data), and found to map very closely to both the FasL and gld loci (1 crossover in 567 meiotic events; Figure 2B). In addition, analysis of YAC clones indicated that the FasL gene was located within an approximately 400 kb segment that contained the gld mutation as defined by flanking crossover events and that the physical distance separating the FasL and Ox40L was less than 300 kb (data not shown). Haplotype

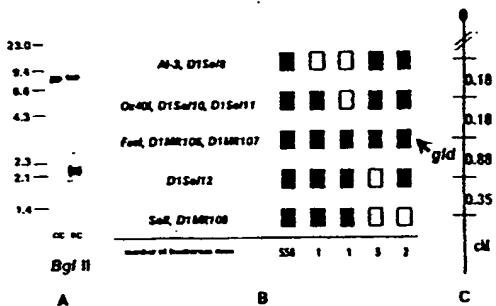


Figure 2. Chromosomal Mapping of the FasL Gene in [(C3H/HeJ-gld × Mus spretus) F1 × C3H/HeJ-gld] Interspecific Backcross Mice  
(A) BglII-digested DNA indicate an RFLP that distinguishes the C3H/HeJ-gld parent (CC) from the (C3H/HeJ-gld × Mus spretus) F1 parent (SC).

(B) Haplotype analyses of the FasL gene demonstrate cosegregation with the gld phenotype on mouse chromosome 1. Gene loci are listed from proximal to distal on the left side. Each column represents a possible haplotype, and the number of mice observed with each haplotype is indicated at the bottom of the column. The boxes indicate whether the mice were typed as C3H/HeJ-gld homozygotes (closed) or F1 heterozygotes (open) for each locus. For RFLP typing of backcross mice, a 1.0 kb 3' cDNA probe spanning base pairs 643 to 1601 and a 5' probe spanning base pairs 1 to 822 were generated by PCR using vector primers. The reference genetic markers on mouse chromosome 1 used for chromosomal mapping studies are described in Experimental Procedures.

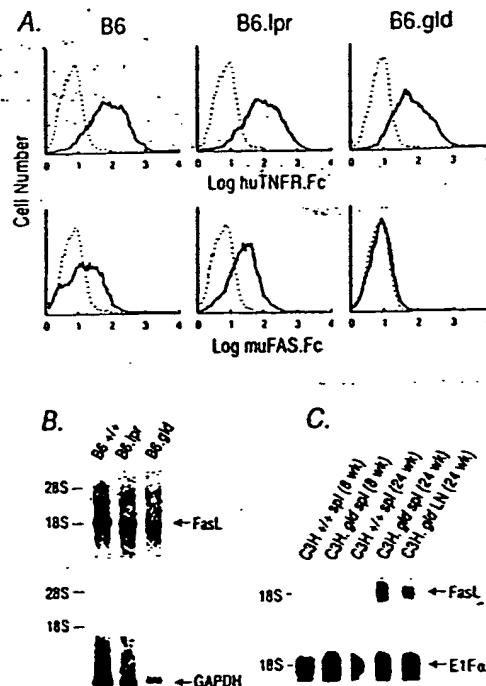
(C) Chromosomal mapping of the murine FasL gene on chromosome 1. The distance between each locus is indicated to the right. The best gene order was determined by multilocus linkage analysis using the BAYLOC algorithm (Blank et al., 1988). Distances between adjacent loci and their standard errors were calculated according to Green (1981).

type analysis indicated the following distances (in centi-Morgans  $\pm$  SEM) among loci mapped to this region of mouse chromosome 1: (centromere) Al-3/D1S108 – 0.18 ( $\pm$  0.18 cM) – Ox40L/D1S101/D1S101 – 0.18 ( $\pm$  0.18 cM) – FasL/D1Mit106/D1Mit107 – 0.88 ( $\pm$  0.39 cM) – D1S102 – 0.35 ( $\pm$  0.25 cM) – SelI/D1Mit108 (Figure 2C).

#### Structure and Function of FasL from gld Mice

To determine whether the gld mutation resulted from a large genomic alteration in the FasL gene, digests of genomic DNA isolated from C3H/HeJ and the coisogenic C3H/HeJgld (C3H-gld) strain mice were compared. Genomic blots of DNAs cut with 19 different 6 base cutting restriction endonucleases and six different 4 base cutting restriction endonucleases were hybridized with FasL gene probes. No differences in band sizes were observed with either the 5' or 3' probes described in Figure 2B, excluding the possibility that any large deletion, insertion, or rearrangement was present in the FasL gene gld mice (data not shown).

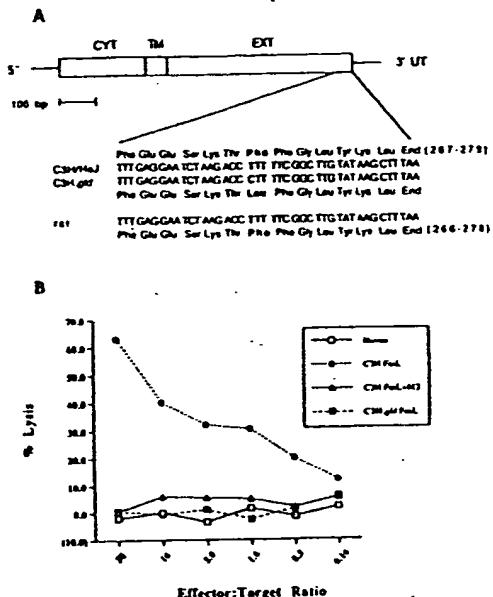
To determine whether the FasL gene was expressed in gld T cells, allospecific CTL of C57BL/6SvJ (B6), B6.MRL/pr (B6.pr), and B6.SMN.C3Hgld (B6.gld) origin were stimulated for 2 hr with phorbol ester and calcium ionophore, and the cell populations were evaluated for cell



**Figure 3. Analysis of *Fas* Expression in T Cells from B6, B6.lpr, and B6.gld Mice**  
(A) FACS analysis of cell surface FasL expression on alloreactive T cells of C57BL/6J (B6), B6.lpr, and B6.gld origin that had been stimulated for 2 hr with phorbol ester and calcium ionophore. Cells were stained with huTNFR(p80).Fc or muFas.Fc fusion proteins (as indicated) to detect cell surface TNF or FasL (solid lines). Aliquots of cells were also stained with hu4L.Fc as a negative control (dotted lines). (B) Northern blot analysis of *Fas*. RNA was extracted from an aliquot of the B6, B6.lpr, and B6.gld T cells evaluated for *Fas* expression in (A). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control for RNA loading. Positions of 18S and 28S RNA are indicated. (C) Northern blot analysis of spleen and lymph node cells from C3H/HeJ and C3H.gld mice. Mouse elongation factor-1 $\alpha$  was used as a control for RNA loading. The position of 18S RNA is indicated.

surface FasL by flow cytometry and for *Fas* mRNA by Northern blot analysis. Although cells of all three genetic backgrounds expressed cell surface TNF as determined by binding of TNFR.Fc, only T cells of B6 and B6.lpr origin expressed cell surface FasL capable of binding Fas.Fc (Figure 3A). However, Northern blot analysis demonstrated similar levels of *Fas* mRNA (as determined by densitometric comparison to control GAPDH mRNA expression) and similar transcript sizes in RNA obtained from all three populations of CTL (Figure 3C).

Expression of *Fas* mRNA was also detected in unstimulated spleen cells from C3H/HeJ and C3H.gld mice. No transcripts of *Fas* were detected in RNA isolated from young C3H/HeJ or C3H.gld mice (Figure 3C). Interestingly, *Fas* transcripts were detected in RNA isolated from



**Figure 4. Structure and Functional Analysis of the *Fas* Gene in Mice Homozygous for the *gld* Mutation**

(A) The predicted structure of mouse *Fas*: CYT, cytoplasmic region; TM, transmembrane region; EXT, extracellular region. The nucleotide sequences and deduced amino acid sequences of the C3H/HeJ, C3H.gld, and rat *Fas* genes at the site of the mutation are shown with the nucleotide and amino acid changes depicted in bold.

(B) The biological activity of FasL\* and FasL\*\* was tested by coculturing COS cells that had been transfected with *Fas* cDNA from wild-type or *gld* mice with  $^{3}\text{H}$ -labeled Jurkat cells, either alone or in the presence of a Fas-specific MAb that specifically inhibits Fas-mediated lytic processes (Harmsdell et al., 1994). COS cells transfected with vector alone served as a negative control. Northern blot analysis of COS cells transfected with *Fas*\* and *Fas*\*\* demonstrated similar levels of expression.

24-week-old C3H.gld mice, but not from wild-type mice. Similar results were obtained with RNA isolated from lymph node cells from these mice. Whether the apparent increase in *Fas* expression in lymphoid cells of older C3H.gld mice is the result of an attempt to compensate for defects in the Fas pathway, or a secondary manifestation of abnormal lymphocyte activation in the course of the autoimmune disease process, is not known at this time.

To identify possible genetic alterations responsible for the *gld* phenotype, the sequence of PCR-generated *Fas* clones from C3H.gld were compared with cDNA clones from both C3H/HeJ and the original C57BL/10J isolate. A single nucleotide difference, a change of a T to C at nucleotide position 847, was detected (Figure 4A). This mutation results in the replacement of a leucine for a phenylalanine residue 7 amino acids from the COOH terminus of this type II transmembrane protein. To determine whether this single amino acid substitution was the basis for the failure of *Fas* to be expressed on the cell surface or to induce apoptosis in Fas-expressing target

cells, COS cells were transfected with *Fas*/cDNA of either wild-type or *gld* origin and tested for *Fas*/ expression by measuring their capacity to bind a soluble Fas.Fc fusion protein as well as their ability to induce lysis of Fas-expressing target cells in a  $^{51}\text{Cr}$  release assay. COS cells transfected with *Fas*/ from wild-type mice bound a soluble Fas.Fc, whereas no binding of Fas.Fc was detected on COS cells transfected with *Fas*/ of *gld* origin (data not shown). In addition, COS cells transfected with *Fas*/cDNA from wild-type mice caused lysis of Jurkat target cells, and this lysis was specifically inhibited by addition of a neutralizing Fas-specific MAb (Figure 4B). In contrast, no lysis of Jurkat target cells was detected using COS cells transfected with *Fas*/ cDNA from *gld* mice. These data indicate that the single base change in the *FasL<sup>gld</sup>* gene alters the functional properties of the ligand.

## Discussion

The cloning of the mouse *Fas*/ gene described here likely represents the single mouse homolog of the rat *Fas*/ described by Suda et al. (Suda et al., 1993). The high degree of both nucleotide and amino acid identity make it unlikely that the gene described in this report is a related family member. High stringency Southern blot analysis also supports a single copy gene with no obvious pseudogenes. Finally, the fact that expression of this gene in COS cells induces Fas-mediated apoptosis of target cells at high levels, and that the target cell lysis was completely inhibited by inclusion of a Fas-specific MAb, confirm that this gene encodes a mouse *Fas*/.

An important aspect of the chromosomal mapping studies was the observation that the mouse *Fas*/ and *Ox40* loci were very tightly linked (1 crossover in 567 meiotic events). The tight linkage of these two TNF family members suggests that a complex of TNF family ligands located on mouse chromosome 1, similar to that previously observed with the TNF, lymphotoxin- $\alpha$  (LT $\alpha$ ), and LT $\beta$  genes on mouse chromosome 17 (Browning et al., 1991, 1993). The close physical relationship between the sequence-related TNF family members on mouse chromosome 17, which are positioned within a relatively small (20 kb) genomic segment, raises the interesting possibility that the mouse chromosome 1 segment surrounding the *Fas*/ also contains additional TNF-like genes.

The possibility that *gld* represents a mutation in the *Fas*/ gene and that *gld* and *lpr* represent a receptor-ligand pair is supported by both previous functional studies of lymphoid cells in *gld* and *lpr* animals (Allen et al., 1990; Ettinger et al., 1993; Ramsdell et al., 1994; Sobel et al., 1993) and structural studies indicating that the ligand for Fas represents a member of the TNF family of molecules (Suda et al., 1993; Watanabe-Fukunaga et al., 1992; Watson et al., 1992b). This report provides both genetic and functional evidence that the *gld* mutation is in the *Fas*/ gene, because it maps to the 0.36 cM/400 kb interval of mouse chromosome 1 that contains the *gld* mutation. The structural and functional studies of the cloned mouse *Fas*/ gene indicate that the *gld* mutation in the *Fas*/ gene, a single nucleotide change that results in the change of a

single amino acid in the carboxy-terminal portion of the molecule, profoundly affects cell surface expression, the ability of *Fas*/ to bind Fas functionally, or both. The result of this failure appears to be a dysregulation of immune responses that leads to the generation of a systemic autoimmune disease process.

## Experimental Procedures

### Experimental Animals

CS7BL/6SnJ (B6), B6.MRL<sub>lpr</sub> (B6.lpr), B6.SMN.C3Hgld (B6.gld), C3H/HeJ, and C3H/HeJgld (C3H.gld) mice were obtained from the Jackson Laboratories, Bar Harbor, Maine.

### Chromosomal Mapping of the *Fas*/ Gene in [(C3H/HeJ-gld × Mus spretus) F1 × C3H/HeJ-gld] Interspecific Backcross Mice

C3H/HeJ-gld and Mus spretus (Spain) parental mice and [(C3H/HeJ-gld × Mus spretus) F1 × C3H/HeJ-gld] interspecific backcross mice were bred and *gld* phenotype positive (presumptive *gld/gld*) or phenotype negative (presumptive *gld/gld*) selected based on three criteria: lymph node and spleen size, serum anti-DNA antibodies, and frequency of CD45 (B220)-positive and surface immunoglobulin-negative spleen cells as previously described (Seldin et al., 1988). DNA was isolated, digested with restriction endonucleases, electrophoresed in 0.9% agarose gels, transferred to nylon membranes, prehybridized, hybridized at 65°C, and washed as previously described (Seldin et al., 1988). The reference genetic markers on mouse chromosome 1 used for chromosomal mapping studies were antithrombin 3 (AT-3), anonymous DNA markers (D1Se18, D1Se17, and D1Se17') and L-selectin (Se1) (Hunter et al., 1993; Seldin et al., 1988; Watson et al., 1992a). The D1Se12 marker was derived by endonuclease recovery from a mouse yeast artificial chromosome (YAC M4-1.gld). For D1Se12, PvuII RFLP distinguished C3H/HeJ-gld (3.5 kb) and Mus spretus (4.1 kb)-derived chromosome segments. All probes were labeled by the hexanucleotide random priming technique (Feinberg and Vogelstein, 1983, 1984) with  $\text{a}^{32}\text{P}$  dCTP. Additional molecular typing was performed using microsatellite markers (D1M106, D1M107, D1M108) obtained from Research Genetics, Huntsville, Alabama, and typed according to the methods of Dietrich et al. The sizes of amplified fragments, D1M106 (C3H/HeJ-gld, 118 bp; Mus spretus, 114), D1M107 (C3H/HeJ-gld, 108 bp; Mus spretus 94) and D1M108 (C3H/HeJ-gld, 148; Mus spretus 155) were used to type backcross mice.

### Northern Blot Analysis

Northern blots were prepared by electrophoresis of 10  $\mu\text{g}$  of total cellular RNA through a 1.1% agarose-formaldehyde gel and blotting to nylon membrane. The Northern blots were hybridized with an anti-sense *Fas*/ riboprobe spanning nucleotides 643 to 1801 and washed at high stringency (0.2 × SSC, 0.1% SDS at 63°C).

### Sequencing

Sequence analysis was performed according to the protocols of the manufacturer using an Applied Biosystems 373A automated sequencer and fluorescein-labeled dye terminator cycle sequencing methods (ABI, Foster City, California). Both strands of PCR-derived templates were sequenced, and multiple independent PCR amplicons were analyzed to avoid Taq polymerase sequencing artifacts. Sequence analyses, including homology searches, were performed using the Wisconsin GCG software package.

### Construction and Production of mυFas.Fc and hυTNFR(p80).Fc Fusion Proteins

A soluble mouse Fas.Fc (m $\mu$ Fas.Fc) fusion protein was constructed by ligating the extracellular domain of the mouse Fas protein to the Fc region of human Immunoglobulin G, (IgG<sub>1</sub>) in the pVL1393 baculovirus transfer vector (PharMingen, San Diego, California). The extracellular domain of mouse Fas was obtained by PCR amplification, using as a template cDNA synthesized from RNA isolated from a mouse T cell clone designated 7C2. The vector was cotransfected with Baculo-Gold Linearized Baculovirus DNA (PharMingen) and recombinant plaques were selected. *Spodoptera frugiperda* 21 cells were infected with re-

combinant virus at a multiplicity of 1 and supernatants containing the muFasFc fusion protein harvested 4 days later. The muFasFc fusion protein was purified from clarified supernatants by affinity chromatography using a protein A-Affigel column (BioRad, Richmond, California). The soluble human TNFR(p80)Fc fusion protein was similarly constructed, expressed, and purified, as previously described (Mohler et al., 1993).

**Indirect Staining with muFasFc for Flow Cytometric Analysis**  
Cells to be evaluated for expression of FasL were first incubated in FACS buffer containing 1% normal mouse serum, 50 µg/ml purified rat anti-mouse FcRγII (2.4G2) to block Fc receptors and minimize non-specific staining, and 0.01% NaN<sub>3</sub> ( $5 \times 10^4$  cells per well in a 96-well microtiter plate) at 4°C in a total volume of 20 µl. Cells were then sequentially incubated with the indicated fusion protein, biotinylated mouse anti-huG (Fc-specific; Jackson Laboratories, West Grove, Pennsylvania), and streptavidin-phycocerythrin (Tago, Burlingame, California). After the final wash, cells were resuspended in 0.3 ml FACS buffer containing 10 ng/ml propidium iodide. Flow cytometry was performed using a FACScan (Becton Dickinson) and data collected on  $1 \times 10^4$  viable cells were analyzed using LYSIS II software.

#### Cytotoxicity Assay

An overnight <sup>51</sup>Cr release assay was used to measure cell lysis induced by FasL. In brief, serial dilutions of COS cells that had been transfected with cDNA from wild-type (C3H/HeJ) FasL or mutant (C3H/gld) FasL were prepared in a 96-well microtiter plate. <sup>51</sup>Cr-labeled Jurkat target cells (5000) were added in 100 µl of medium. After overnight culture at 37°C, plates were centrifuged (150 × g for 5 min) and supernatants harvested using a Skatron SCS harvesting system (Skatron, Sterling, Virginia). <sup>51</sup>Cr content of supernatants was determined using a Micromedic ME Plus gamma counter (Micromedics, Huntsville, Tennessee). Percent specific <sup>51</sup>Cr content of supernatants was calculated according to the formula:  $100 \times [(\text{experimental cpm}) - (\text{spontaneous cpm})] / [(\text{maximum cpm}) - (\text{spontaneous cpm})]$ , where spontaneous cpm = cpm released in medium alone and maximum cpm = cpm released in the presence of 1N HCl.

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**Note Added in Proof**

Subsequent to the submission of this manuscript, an independent report by Takahashi et al. (Cell 76, 969-976) has also shown that the *gld* mouse contains a single base permutation in *Fas*.

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